Chapter six

Summary and general conclusions

Numerous microorganisms, both prokaryotes and eukaryotes, have developed various strategies, which enable them to adapt and survive the often adverse circumstances present in their natural environment. One such adaptation is the production of compounds to facilitate the uptake of essential metal ions and to inhibit competing organisms. Often these compounds are of peptide origin, synthesized either ribosomally or nonribosomally. Because many of these peptides exhibit antibiotic or other medically interesting properties, a growing interest in these peptides has developed in recent years.

Until now, more than 300 different precursors, including pseudo, nonproteinogenic, hydroxy, *N*-methylated and D-amino acids have been identified in nonribosomally synthesized peptides. The peptide backbone of these peptides can be composed of linear, cyclic, or cyclic branched structures, which can be further modified by acylation, glycosylation, or heterocyclic ring formation. Because large quantities of energy are invested in their biosynthesis, in nature the nonribosomally synthesized peptides probably play an important role in the struggle for survival, and a large spectrum of activities has been observed. Often nonribosomally synthesized peptides are surface active and may exhibit antibiotic, antifungal, antitumor or antiviral activities. In addition, various nonribosomally synthesized peptides exhibit enzyme-inhibiting or immunosuppressive activities.

The biosynthesis of these peptides is performed by large multienzyme complexes, designated peptide synthetases, which exhibit a modular structure. A module is defined as the minimal independent unit that catalyzes all the necessary reactions to modify and incorporate one specific amino acid into the growing peptide chain. The genetic arrangement of the genes encoding these modules is usually colinear with the amino acid sequence of the peptide product (colinearity rule). The modules can be further subdivided into domains catalysing the various reactions necessary to incorporate an amino acid into the growing peptide. All modules consist of an adenylation domain for amino acid activation, a thiolation domain essential for the covalent binding of the activated amino acid, and a condensation domain performing the coupling of two subsequently bound amino acids. In addition, domains can be present modifying the covalently bound amino acids prior to the peptide bond formation, such as an epimerization domain, converting the bound amino acid from the L configuration to the D configuration or an N-methylation domain, methylating nitrogen atoms in the bound amino acid. Also a thioesterase is present in all peptide synthetases, either as a C-terminally integrated domain or as a separate protein, which is involved in cyclization and release of the synthesized product. In recent years an increasing number of peptide synthetases have been discovered, which contain modules previously identified in fatty acid- or polyketide synthases. These hybrid synthetases not only incorporate amino acids, but also various building blocks present in fatty acids and polyketides.

The operons encoding peptide synthetases can be as large as 50 kb and are, together with the operons encoding polyketide synthases, the largest operons present on the genomes of microorganisms. The colinearity rule mentioned earlier not only applies to the modular structure of the peptide synthetases at the translational level but usually also to the structural organization of the peptide synthetase operons. The order of the genes encoding the subunits of the synthetase reflects the order of peptide synthesis. Some genes, encoding proteins performing essential enzymatic steps, are not present in peptide synthetase operons. This concerns, for instance, the genes encoding the 4'-phosphopantetheine transferases, which couple this essential cofactor to the thiolation domains, and genes encoding enzymes involved in the synthesis of precursors.

Relatively little is known of the transcriptional regulation of the peptide synthetase operons with the exception of the expression and transcriptional regulation of the surfactin synthetase operon, *srfA*, in *B. subtilis*, which has been studied rather extensively. Expression of this operon is induced at the transition from exponential to stationary growth when nutrients become limiting, and is controlled by the interactions of regulatory proteins, which also function in controlling other stationary-phase-induced processes, like sporulation and the development of genetic competence. Expression of *srfA* is under cell density control and dependent on the activity of a two-component regulatory system, ComA and ComP. Phosphorylated ComA, which phosphorylation state is determined by the activity of the kinase ComP and the phosphatase RapC, induces expression of this operon. Expression of *srfA* is also controlled by the proteins Spo0H, Spo0K and PhrC, which are involved in regulation of the activity of RapC. Finally, the nutrition dependent repressor, CodY, is also involved in transcriptional regulation of *srfA*.

Little is also known about the proteins that facilitate secretion and mediate self-resistance. Mostly this is performed by ABC-transporters, which couple ATP hydrolysis to transport across the cell membrane, or by membrane proteins, which energize the transport across the membrane by proton-dependent transmembrane electrochemical gradients. Only in a few cases ABC-transporters have been identified that are involved in secretion and self-resistance. In all these cases the genes encoding these proteins were located in or close to the corresponding operons.

The research presented in this thesis concerns nonribosomally synthesized peptides of the gram-positive bacterium *B. subtilis*, a well known producer of mainly cyclic lipopeptides.

Chapter 2 describes for the first time the identification, sequence determination and initial analysis of the mycosubtilin synthetase operon, *mycS*, a hybrid synthetase. As expected biochemical characterization showed that the first module of MycB specifically adenylates tyrosine. Insertional mutagenesis of the operon resulted in a mycosubtilin-negative phenotype. These results clearly demonstrate that the identified operon encodes the mycosubtilin synthetase. The operon spans about 38 kb and consists of four genes, *fenF*, *mycA*, *mycB* and *mycC*. The proteins MycA, MycB and MycC together form the mycosubtilin synthetase. The first protein, FenF, is highly homologous to malonyl-CoA transacylases. The mycosubtilin synthetase operon is present on the genome of *B. subtilis* ATCC6633 at a position identical to that of the fengycin synthetase operon in *B. subtilis*

168, suggesting that the fengycin and mycosubtilin synthetase operons were exchanged between the two *B. subtilis* strains. Except for some minor differences analysis of the organization of the synthetase subunits showed it to be comparable to that found in other peptide synthetases. However, the first subunit of the mycosubtilin synthetase, MycA, combines functional domains present in peptide synthetases as well as fatty acid or polyketide synthases, and a domain homologous to amino transferases. This unique combination of domains made it the first hybrid synthetase discovered and analyzed. Finally, a model for the biosynthesis of mycosubtilin is presented, based on characteristics of the mycosubtilin synthetase and reactions performed by homologous enzymes. This model presumes that the first five domains of MycA are involved in the conversion of a fatty acid to a β-amino fatty acid after which peptide synthesis is continued as in other peptide synthetases synthesising lipopeptides. The modular structure of peptide synthetases will allow the engineering of novel peptide synthetases by means of domain swapping, which is the exchange of adenylation domains of different modules resulting in the synthesis of novel peptides.

Chapter 3 describes the development of two methods that enabled the analysis of the expression and transcriptional regulation of the mycosubtilin synthetase operon, *mycS*. Due to low levels of genetic competence *B. subtilis* ATCC6633 is extremely difficult to transform. This and unstable maintenance of plasmids containing the *mycS* promoter region in *E. coli*, severely hampered genetic studies of *mycS*. To increase the levels of genetic competence and thus the transformation efficiency, the low-copy plasmid pGSP12, containing the gene encoding the competence transcription factor ComK under the control of its own promoter, was used. Introduction of this plasmid into *B. subtilis* ATCC6633 and *B. subtilis* ATCC21332, using protoplast transformation, increased the transformation efficiency of these strains about 250 fold and 70 fold, respectively.

The second method facilitates Campbell-type genomic integrations in *B. subtilis* by using DNA ligation mixtures, and bypasses the need of subcloning in *E. coli*. Addition of polyethyleenglycol 8000 (PEG_{8000}) to the ligation mixture causes macromolecular crowding, which strongly stimulates the formation of large linear multimeric ligation products that appeared to be efficient substrates for Campbell-type integrations into competent *B. subtilis* cells.

The study described in Chapter 4 revealed that expression of *mycS* and *srfA* in *B. subtilis* ATCC6633 is several hundred fold lower than that of *srfA* in *B. subtilis* 168. In contrast to the activity of the *srfA* promoter, the activity of the *mycS* promoter in the *B. subtilis* 168 did not differ from that in *B. subtilis* ATCC6633. In contrast to *srfA*, which showed highest expression in minimal medium, *mycS* showed highest expression in TY-medium. The transcriptional regulation of *srfA* in *B. subtilis* ATCC6633 appeared to be comparable to that in *B. subtilis* 168, and was mainly dependent on ComA. Expression of *mycS* in *B. subtilis* ATCC6633 and *B. subtilis* 168 was found to be independent of ComA, but still seemed to be regulated via quorum sensing as addition of PhrC to the culture medium strongly stimulated expression and bypassed the effects of a *spo0H* deletion. Finally, deletion of *abrB* resulted in increased expression of *mycS* in *B. subtilis* ATCC6633 demonstrating that this pleitropic transition state regulator repressed expression of this operon. However, as the expression of *mycS* in the *abrB* deletion strain was still

temporal additional proteins must be involved in the transcriptional regulation of this operon.

Chapter 5 describes the effects of the lipopeptide surfactin, produced by various *B. subtilis* strains, on phosphatidylcholine bilayers. Surfactin partially induced carboxyfluorescein (CF) leakage from loaded vesicles in a pH dependent way, but was almost completely inactive in dissipating an induced $\Delta\psi$, either inside positive or inside negative. Hemolytic activity was also found to be pH dependent, and both induced CF leakage as well as the hemolytic activity were strongest at pH 6.0 and became weaker at higher pH values. Induction of an inside positive $\Delta\psi$ stimulated surfactin-induced CF leakage at pH 7.0. Only little stimulation was observed at pH 6.0 and pH 8.0. An inside negative $\Delta\psi$ did not significantly influence the surfactin induced CF leakage from loaded vesicles.

In general, this thesis provides more insight in nonribosomal peptide synthesis in *B. subtilis*. The discovery of a hybrid synthetase opens the possibility to combine domains of peptide synthetases and fatty acid or polyketide synthases by means of domain swapping, which has been proven to be possible for peptide synthetases as well as fatty acid or polyketide synthases.

The developed methods enable genetic studies on *B. subtilis* strains exhibiting no or insignificant levels of genetic competence and genes that are unstable maintained in *E. coli*.

As increasing evidence is obtained demonstrating that peptide synthetases are essential for virulence of various pathogenic bacteria and fungi, the study of their regulation may be helpful in understanding their role in pathogenesis.

In the absence of any unequivocal data on the existence of a specific secretion mechanism for surfactin, it is conceivable that this lipopeptide is spontaneously secreted without facilitation of a specialized protein. Whether this is true requires a considered extension of the research presented in this thesis.

Finally, this thesis may contribute to the ultimate goal of most research carried out in this area: the development of new medicines by means of nonribosomal peptide synthetases.